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(54) Title: DNA CONSTRUCTS AND PLANTS INCORPORATING THEM

(57) Abstract

The invention relates to a chemically-inducible plant gene expression cassette and to plant cells transformed therewith. The expression cassette is suitable for use in any dicothyledonous or monocotyledonous plant. It comprises a first promoter operatively linked to a regulator sequence which encodes a regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene. The regulator sequence may be derived from the *alc*R gene, and the inducible promoter may be derived from the *alc*A gene promoter (both obtainable from *Aspergillus nidulans*) A chimeric promoter comprising an upstream regulatory region and a heterologous downstream transcription initiation region is also disclosed. The regulatory sequence may be derived from the *alc*A gene promoter.

DNA CONSTRUCTS AND PLANTS INCORPORATING THEM

The present invention relates to DNA
constructs and plants incorporating them. In
particular, it relates to expression cassettes and
promoter sequences for the expression of genes in
plants.

Gene expression is controlled by regions
upstream (5') of the protein encoding region,
commonly referred to as the "promoter". A promoter
may be constitutive, tissue-specific,
developmentally-programmed or inducible.

Manipulation of crop plants to improve
characteristics (such as productivity or quality)
requires the expression of foreign or endogenous
genes in plant tissues. Such genetic manipulation
therefore relies on the availability of means to
control gene expression as required; for example,
on the availability and use of suitable promoters
which are effective in plants. It is advantageous
to have the choice of a variety of different
promoters so that the most suitable promoter may be
selected for a particular gene, construct, cell,
tissue, plant or environment. A range of promoters
are known to be operative in plants.

Within the promoter region there are several
domains which are necessary for full function of
the promoter. The first of these domains lies
immediately upstream of the structural gene and
forms the "core promoter region" containing
consensus sequences, normally 70 base pairs

immediately upstream of the gene. The core
promoter region contains the characteristic CAAT
and TATA boxes plus surrounding sequences, and
represents a transcription initiation sequence
5   which defines the transcription start point for the
structural gene. The precise length of the core
promoter region is indefinite but it is usually
well-recognisable. Such a region is normally
present, with some variation, in all promoters.
10   The base sequences lying between the various
well-characterised "boxes" appear not to be of
great importance.

       The presence of the core promoter region
15   defines a sequence as being a promoter: if the
region is absent, the promoter is non-functional.
Furthermore, the core promoter region is
insufficient to provide full promoter activity. A
series of regulatory sequences upstream of the core
20   constitute the remainder of the promoter. The
regulatory sequences determine expression level,
the spatial and temporal pattern of expression and,
for an important subset of promoters, expression
under inductive conditions.
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       Several naturally-occurring promoters and
associated gene expression systems are known. The
best characterised regulatory systems are those of
bacteria in which the specific interactions between
30   DNA-binding proteins (repressors) and the target
DNA sequences (operators) results in the negative
repression of gene activity.

       The alcA/alcR gene activation system from the

fungus <u>Aspergillus</u> <u>nidulans</u> is also well
characterised. The ethanol utilization pathway in
<u>A</u> <u>nidulans</u> is responsible for the degradation of
alcohols and aldehydes. Three genes have been
5   shown to be involved in the ethanol utilization
pathway. Genes <u>alcA</u> and <u>alcR</u> have been shown to
lie close together on linkage group VII and <u>aldA</u>
maps to linkage group VIII (Pateman JH et al, 1984,
Proc. Soc. Lond, B217:243-264; Sealy-Lewis HM and
10   Lockington RA, 1984, Curr. Genet, 8:253-259). Gene
<u>alcA</u> encodes ADHI in <u>A</u> <u>nidulans</u> and <u>aldA</u> encodes
AldDH, the second enzyme responsible for ethanol
utilization. The expression of both <u>alcA</u> and <u>aldA</u>
are induced by ethanol and a number of other
15   inducers (Creaser EH et al, 1984, Biochemical J,
255:449-454) via the transcription activator <u>alcR</u>.
The <u>alcR</u> gene and a co-inducer are responsible for
the expression of <u>alcA</u> and <u>aldA</u> since a number of
mutations and deletions in <u>alcR</u> result in the
20   pleiotropic loss of ADHI and aldDH (Felenbok B et
al, 1988, Gene, 73:385-396; Pateman et al, 1984;
Sealy-Lewis & Lockington, 1984). The ALCR protein
activates expression from <u>alcA</u> by binding to three
specific sites in the <u>alcA</u> promoter (Kulmberg P et
25   al, 1992, J. Biol. Chem, 267:21146-21153).

The <u>alcR</u> gene was cloned (Lockington RA et al,
1985, Gene, 33:137-149) and sequenced (Felenbok et
al, 1988). The expression of the <u>alcR</u> gene is
30   inducible, autoregulated and subject to glucose
repression mediated by the CREA repressor (Bailey C
and Arst HN, 1975, Eur. J. Biochem, 51:573-577;
Lockington RA et al, 1987, Mol. Microbiology,
1:275-281; Dowzer CEA and Kelly JM, 1989, Curr.

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Genet, 15:457-459; Dowzer CEA and Kelly JM, 1991,
Mol. Cell. Biol, 11:5701-5709). The ALCR
regulatory protein contains 6 cysteines near its N
terminus coordinated in a zinc binuclear cluster
5   (Kulmberg P et al, 1991, FEBS Letts, 280:11-16).
This cluster is related to highly conserved DNA
binding domains found in transcription factors of
other ascomycetes. Transcription factors GAL4 and
LAC9 have been shown to have binuclear complexes
10   which have a cloverleaf type structure containing
two Zn(II) atoms (Pan T and Coleman JE, 1990,
Biochemistry, 29:3023-3029; Halvorsen YDC et al,
1990, J. Biol. Chem, 265:13283-13289). The
structure of ALCR is similar to this type except
15   for the presence of an asymmetrical loop of 16
residues between Cys-3 and Cys-4. ALCR positively
activates expression of itself by binding to two
specific sites in its promoter region (Kulmberg P
et al, 1992, Molec. Cell. Biol, 12:1932-1939).

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The regulation of the three genes, alcR, alcA
and aldA, involved in the ethanol utilization
pathway is at the level of transcription
(Lockington et al, 1987; Gwynne D et al, 1987,
25   Gene, 51:205-216; Pickett et al, 1987, Gene,
51:217-226).

There are two other alcohol dehydrogenases
present in A nidulans. ADHII is present in mycelia
30   grown in non-induced media and is repressible by
the presence of ethanol. ADHII is encoded by alcB
and is also under the control of alcR (Sealy-Lewis
& Lockington, 1984). A third alcohol dehydrogenase
has also been cloned by complementation with a adh-

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strain of S cerevisiae. This gene alcC, maps to
linkage group VII but is unlinked to alcA and alcR.
The gene, alcC, encodes ADHIII and utilizes ethanol
extremely weakly (McKnight GL et al, 1985, EMBO J,

5   4:2094-2099). ADHIII has been shown to be involved
in the survival of A nidulans during periods of
anaerobic stress. The expression of alcC is not
repressed by the presence of glucose, suggesting
that it may not be under the control of alcR

10   (Roland LJ and Stromer JN, 1986, Mol. Cell. Biol,
6:3368-3372).

In summary, A nidulans expresses the enzyme
alcohol dehydrogenase I (ADH1) encoded by the gene

15   alcA only when it is grown in the presence of
various alcohols and ketones. The induction is
relayed through a regulator protein encoded by the
alcR gene and constitutively expressed. In the
presence of inducer (alcohol or ketone), the

20   regulator protein activates the expression of the
alcA gene. The regulator protein also stimulates
expression of itself in the presence of inducer.
This means that high levels of the ADH1 enzyme are
produced under inducing conditions (ie when alcohol

25   or ketone are present). Conversely, the alcA gene
and its product, ADH1, are not expressed in the
absence of inducer. Expression of alcA and
production of the enzyme is also repressed in the
presence of glucose.

30
Thus the alcA gene promoter is an inducible
promoter, activated by the alcR regulator protein
in the presence of inducer (ie by the
protein/alcohol or protein/ketone combination).

The alcR and alcA genes (including the respective
promoters) have been cloned and sequenced
(Lockington RA et al, 1985, Gene, 33:137-149;
Felenbok B et al, 1988, Gene, 73:385-396; Gwynne et
5   al, 1987, Gene, 51:205-216).

Alcohol dehydrogenase (adh) genes have been
investigated in certain plant species. In maize
and other cereals they are switched on by anaerobic
10   conditions. The promoter region of adh genes from
maize contains a 300 bp regulatory element
necessary for expression under anaerobic
conditions. However, no equivalent to the alcR
regulator protein has been found in any plant.
15   Hence the alcR/alcA type of gene regulator system
is not known in plants. Constitutive expression of
alcR in plant cells does not result in the
activation of endogenous adh activity.

20   The knowledge of mechanisms by which gene
expression is regulated in eukaryotes is much less
detailed than the knowledge of bacterial systems.
In yeast and mammalian cells a large number of
binding sites for putative regulatory proteins have
25   been identified in promoter sequences, and in some
cases the proteins responsible have also been
isolated. However, only in a few instances are the
molecular details known of the protein-DNA
interactions and the mechanism by which
30   transcription is regulated. In plants, regulation
of gene expression is understood at only a
rudimentary level. Several regulatory elements
have been identified in promoter sequences, and
some regulatory proteins examined at a preliminary

level. Many such proteins have yet to be isolated and the details of the mechanisms involved have yet to be elucidated.

5   The control of expression of heterologous genes in plant tissues is important for successful genetic manipulation of plants to alter and/or improve phenotypic characteristics. Promoters and/or regulatory components from bacteria,
10   viruses, fungi and plants have been used to control gene expression in plant cells. For example, two well-characterised bacterial operator-repressor systems have been used to show negative (down) regulation of gene expression in plant cells. A
15   modified bacterial tet operator-repressor system has been shown to repress gene expression in electroporated plant cells. Gene expression from the CAT gene was reduced by the placing of tet operators in the 35S promoter (Gatz and Quail,
20   1988, Proc. Natl. Acad. Sci. USA, 85:1394-1397). Functional tet repressor expressed from an integrated gene also represses expression from an integrated β-glucuronidase gene (gus) driven by the 35S promoter containing two inserted tet
25   operators (Gatz et al, 1991, Mol. Gen. Genet, 227:229-237).

       The lac operator-repressor system has been used to repress gene expression in tobacco cells.
30   Functional LacI protein expressed from a stably integrated lacI gene repressed expression from a stably integrated gus gene controlled by the maize chlorophyll a/b binding protein (CAB) promoter containing a lac operator. Derepression of gus

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activity occurred when the cells were incubated
with the inducer iso-propyl thiogalactoside (IPTG)
showing that repression of gene activity is
specifically due to the lacI gene product (Wilde RJ
5   et al, 1992, EMBO J, 11:1251-1259).

As stated above, successful genetic
manipulation relies on the availability of means to
control gene expression as required. The scientist
10   may use a suitable expression cassette
(incorporating one or more promoters and other
components) to regulate gene expression in the
desired manner. The ability to enhance or reduce
gene expression to achieve a desired phenotypic
15   effect according to external circumstances is
particularly advantageous. We now provide a novel
plant gene expression cassette which allows
external activation of target gene expression
within plants.
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According to a first aspect of the invention,
there is provided a chemically-inducible plant gene
expression cassette comprising a first promoter
operatively linked to a regulator sequence which
25   encodes a regulator protein, and an inducible
promoter operatively linked to a target gene, the
inducible promoter being activated by the regulator
protein in the presence of an effective exogenous
inducer whereby application of the inducer causes
30   expression of the target gene.

The first promoter may be constitutive, or
tissue-specific, developmentally-programmed or even
inducible. The regulator sequence is preferably

the alcR gene obtainable from Aspergillus nidulans,
encoding the alcR regulator protein.

The inducible promoter is preferably the alcA
gene promoter obtainable from Aspergillus nidulans,
or a "chimeric" promoter derived from the
regulatory sequences of the alcA promoter and the
core promoter region from a gene promoter which
operates in plant cells (including any plant gene
promoter). The alcA promoter or a related
"chimeric" promoter is activated by the alcR
regulator protein when an alcohol or ketone inducer
is applied.

The inducible promoter may also be derived
from the aldA gene promoter, the alcB gene promoter
or the alcC gene promoter obtainable from
Aspergillus nidulans.

The target gene may be an endogenous plant
gene or a foreign gene, and may be a single gene or
a series of genes. The target gene sequence
encodes at least part of a functional protein or an
antisense sequence.

The inducer may be any effective chemical
(such as an alcohol or ketone). Suitable chemicals
for use with an alcA/alcR-derived cassette include
those listed by Creaser et al (1984, Biochem J,
225, 449-454) such as butan-2-one (ethyl methyl
ketone), cylcohexanone, acetone, butan-2-ol,
3-oxobutyric acid, propan-2-ol, ethanol.

The gene expression cassette is responsive to

an applied exogenous chemical inducer enabling
external activation of expression of the target
gene regulated by the cassette. The expression
cassette is highly regulated and suitable for
5   general use in plants.

     The two parts of the expression cassette may
be on the same construct or on separate constructs.
The first part comprises the regulator cDNA or gene
10   sequence subcloned into an expression vector with a
plant-operative promoter driving its expression.
The second part comprises at least part of an
inducible promoter which controls expression of a
downstream target gene. In the presence of a
15   suitable inducer, the regulator protein produced by
the first part of the cassette will activate the
expression of the target gene by stimulating the
inducible promoter in the second part of the
cassette.
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     As an example of the invention, a gene
expression cassette was constructed comprising the
Aspergillus nidulans alcR gene under control of a
constitutive promoter and an alcA:35S inducible
25   "chimeric" promoter linked to the chloramphenicol
acetyl transferase (CAT) gene from E coli.
Constructs representing the two parts of this
expression cassette were tested in Aspergillus
nidulans. For example:
30   (1) An alcR cDNA was expressed in A nidulans by
          placing it downstream from the CaMV 35S or nos
          promoter. Transformants of an alcR⁻ strain
          were shown to be wild type (alcR⁺) with
          respect to growth on ethanol as sole carbon

source, showing that the alcR construct was
expressing the regulator protein.

(2) A construct containing a CAT gene downstream
from the alcA:35S "chimeric" promoter was

5   tested in a (alcR$^+$) A nidulans strain. The
CAT gene was used as the target gene because
it is a suitable reporter gene for monitoring
gene expression. CAT activity was
alcohol-inducible (ie repressor protein plus

10   inducer were activating the "chimeric"
promoter), showing that the "chimeric"
promoter is functional.

.(3) The regulator alcR construct and the alcA
reporter construct were co-transformed into an

15   alcR$^-$A$^-$ deletion strain. Any expression of
the CAT reporter gene is induced by the alcR
regulator protein produced by the regulator
construct, without interference from a
wild-type regulator protein. The alcA:35S

20   chimeric promoter is inducible: the promoter
drives expression of the CAT reporter gene
when the regulator protein and inducer are
present. Thus the gene expression cassette
(comprising the alcR and alcA constructs) is

25   functional and chemically-inducible (ie
subject to external activation).

This invention is based on the construction of
the above type of expression cassette and on our

30   recognition that the functional elements of the
cassette are suitable for application in other
organisms, particularly in plants (such as tobacco,
tomato, canola, sugarbeet, sunflower, maize, or
wheat).

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In practice the construct or constructs comprising the expression cassette of the invention will be inserted into a plant by transformation. Expression of target genes in the construct, being

5   under control of the chemically switchable promoter of the invention, may then be activated by the application of a chemical inducer to the plant.

Any transformation method suitable for the

10   target plant or plant cells may be employed, including infection by Agrobacterium tumefaciens containing recombinant Ti plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and

15   pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be

20   obtained in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco,

25   sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

30   The invention further provides a plant cell containing a gene expression cassette according to the invention. The gene expression cassette may be stably incorporated in the plant's genome by transformation. The invention also provides a

plant tissue or a plant comprising such cells, and
plants or seeds derived therefrom.

5   The invention further provides a method for
      controlling plant gene expression comprising
      transforming a plant cell with a
      chemically-inducible plant gene expression cassette
      which has a first promoter operatively linked to a
      regulator sequence which encodes a regulator
10   protein, and an inducible promoter operatively
      linked to a target gene, the inducible promoter
      being activated by the regulator protein in the
      presence of an effective exogenous inducer whereby
      application of the inducer causes expression of the
15   target gene.

      As an example of the invention, we have
      demonstrated the functioning of alcR/alcA
      constructs in plant cells, and have shown that the
20   system is useful as an inducible plant gene
      expression cassette. Regulator and reporter
      constructs were transformed separately and together
      into maize plant protoplasts for transient gene
      expression assays. Expression of the CAT gene from
25   the alcA promoter in maize protoplasts incubated in
      the presence of ethanol (inducer) is dependent on
      the presence of the alcR gene (expressing the
      regulator protein). Stably transformed tobacco
      plants containing the alcR/alcA-CAT expression
30   cassette were also produced. High levels of
      reporter (CAT) gene expression were only obtained
      in the presence of the alcR regulator gene product
      and following chemical induction by cyclohexanone.
      High levels of activity from the alcA-CAT reporter

gene construct were <u>not</u> obtained under a range of
growth conditions such as anaerobis, indicating
that reporter gene expression was not induced by
conditions that might normally be expected to
5   induce plant alcohol dehydrogenases.

The inducible promoter forming part of the
gene expression cassette according to the invention
may be a "chimeric" promoter sequence, created as
10   described above by fusing heterologous upstream and
downstream regions. Such a chimeric promoter may
have independent utility as an alternative plant
promoter for use in genetic manipulation. Comai <u>et</u>
<u>al</u> have previously described a chimeric plant   .
15   promoter combining elements of the CaMV35S and the
mannopine synthase (<u>mas</u>) promoters (1990, Plant Mol
Biol, 15:373-381). In electroporated tobacco
cells, the yeast GAL4 protein has been shown to
stimulate CAT gene expression controlled from a 35S
20   promoter which had GAL4 binding sites upstream from
the TATA box (Ma J et al, 1988, Nature, 334:631-
633).

According to a second aspect of the invention,
25   there is provided a chimeric promoter comprising an
upstream region containing a promoter regulatory
sequence and a downstream region containing a
transcription initiation sequence, characterised in
that said upstream and downstream regions are
30   heterologous.

The upstream region may contain a
constitutive, a tissue-specific, a
developmentally-programmed or an inducible promoter

regulatory sequence. There may be one or more
regulatory sequences in the upstream region. The
downstream region is a core promoter region. The
upstream and downstream regions correspond to
5   sequences isolated respectively from different
sources. The upstream or downstream regions are
fused together. The upstream and/or downstream
regions may be synthesised.

10   The upstream sequence is preferably derived
from the inducible alcA gene promoter obtainable
from Aspergillus nidulans. The upstream sequence
may also be derived from the aldA, alcB or alcC
gene promoters obtainable from Aspergillus
15   nidulans. The downstream sequence may be derived
from the core promoter region of any
plant-operative promoter (such as the CaMV35S
promoter, MFS14, MFS18, GSTII-27, pMR7,
Polygalacturonase promoters), or may be synthesised
20   from consensus sequences.

        The chimeric promoter may be operatively
linked to one or more target gene sequences
encoding at least part of a functional protein or
25   an antisense RNA. The target gene may be any
endogenous plant gene or any foreign gene.

        In practice the promoter of the invention will
be inserted as a promoter sequence in a recombinant
30   gene construct destined for use in a plant. The
construct will then be inserted into the plant by
transformation. Any plant species may be
transformed with the construct, and any suitable
transformation method may be employed.

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The invention further provides a plant cell
containing a chimeric promoter according to the
invention. The chimeric promoter may be stably
incorporated in the plant's genome by
5   transformation. The invention also provides a
plant tissue or a plant comprising such cells, and
plants or seeds derived therefrom.

As an example of the invention, a chimeric
10   gene promoter sequence has been produced by fusing
a regulatory region from the Aspergillus nidulans
alcA gene promoter to part of the −70 core region
of the Cauliflower mosaic virus (CaMV) 35S promoter
(see Example 2 and Figure 3). The alcA:35S
15   chimeric promoter sequence was linked to the
chloramphenicol acetyl transferase (CAT) gene from
E coli and transformed into an (alcR⁺) A nidulans
strain. The CAT gene was used as a reporter gene
to monitor gene expression. CAT production
20   indicated that the chimeric promoter was
functioning correctly (see Example 3).

The alcA:35S chimeric promoter is also
suitable for use in any plant. Transient
25   expression assays in maize protoplasts (Example 4)
and expression from stably-incorporated genes in
tobacco (Example 5) have shown that the alcA:35S
promoter is fully functional in plant cells.

30   The invention will now be described by way of
example only with reference to the accompanying
drawings in which:
Figure 1 shows a scheme for the production of
the 35S regulator construct.

Figure 2 shows a scheme for the production of the pNOS regulator construct.

Figure 3 shows the production of the reporter construct.

5   Figure 4 is a graph showing CAT expression from A nidulans alcA-CAT transformants.

Figure 5 is a graph showing CAT expression from an A nidulans alcA-CAT transformant against cyclohexanone concentration.

10   Figure 6 is a graph showing CAT expression from an A nidulans alcA-CAT transformant and a 35S-CAT transformant.

Figure 7 is a graph showing relative CAT expression levels in maize protoplasts.

15   Figure 8 is a graph showing relative CAT expression levels in maize protoplasts.

Figure 9 is a graph showing relative CAT expression levels in stably transformed tobacco.

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EXAMPLE 1
PRODUCTION OF THE alcR REGULATOR CONSTRUCT.

25   The alcR genomic DNA sequence has been published, enabling isolation of a sample of alcR cDNA.

The alcR cDNA was cloned into two different
30   expression vectors, pJR1(pUC) and pNOS. pJR1 contains the Cauliflower Mosaic Virus 35S promoter and pNOS contains the nos gene promoter from the Ti Plasmid of Agrobacterium tumefaciens. Both of these promoters are constitutive plant promoters

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and will continually express the regulator protein.
The nos polyadenylation signal is used in both the
expression vectors.

5   Figure 1 illustrates the production of the
35S regulator construct by ligation of alcR cDNA
into pJR1. Partial restriction of the alcR cDNA
clone with BamHI was followed by electrophoresis in
an agarose gel and the excision and purification of
10   a 2.6 Kb fragment. The fragment was then ligated
into the pJR1 vector which had been restricted with
BamHI and phosphatased to prevent
recircularisation. The alcR gene was thus placed
under control of the CaMV 35S promoter and the nos
15   3' polyadenylation signal in this "35S-alcR"
construct.

      Figure 2 illustrates the production of the
pNOS regulator construct by ligation of alcR cDNA
20   into pNOS. Restriction of the alcR cDNA clone with
EcoRI and SalI was followed by electrophoresis and
the excision and purification of a 2.8 Kb fragment.
The pNOS vector was restricted with EcoRI and XhoI,
phosphatased and then ligated to the 2.8 Kb alcR
25   fragment. The alcR gene was thus placed under the
control of the nos gene promoter and the nos 3'
polyadenylation signal in this "nos-alcR"
construct.

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EXAMPLE 2

PRODUCTION OF THE alcA-CAT REPORTER CONSTRUCT CONTAINING THE CHIMERIC PROMOTER.

The plasmid pCaMVCN contains the bacterial
5   chloramphenicol transferase (CAT) reporter gene
between the 35S promoter and the nos transcription
terminator (the "35S-CAT" construct).

The alcA promoter was subcloned into the
10   vector pCaMVCN to produce an "alcA-CAT" construct.
Fusion of part of the alcA promoter and part of the
35S promoter created a chimeric promoter which
allows expression of genes under its control.

15   Figure 3 illustrates the production of the
reporter construct. The alcA promoter and the 35S
promoter have identical TATA boxes which were used
to link the two promoters together using a
recombinant PCR technique: a 246 bp region from the
20   alcA promoter and the 5' end of the CAT gene from
pCaMVCN (containing part of the -70 core region of
the 35S promoter) were separately amplified and
then spliced together using PCR. The recombinant
fragment was then restriction digested with BamHI
25   and HindIII. The pCaMVCN vector was partially
digested with BamHI and HindIII, then
electrophoresed so that the correct fragment could
be isolated and ligated to the recombinant
fragment.
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The ligation mixtures were transformed into E
coli and plated onto rich agar media. Plasmid DNA
was isolated by miniprep from the resultant
colonies and recombinant clones were recovered by

size electrophoresis and restriction mapping. The
ligation junctions were sequenced to check that the
correct recombinants had been recovered.

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EXAMPLE 3

EXPRESSION IN ASPERGILLUS NIDULANS.

The completed alcR and alcA constructs were
transformed into A nidulans to test their

10   functioning. Integration of the constructs into
the genome is verified by Southern blotting of DNA
fragments, while correct expression is shown by
northern blotting to identify RNA transcripts.

15   Growth conditions

A nidulans was maintained on complete or
minimal media containing 1 % agar. A nidulans
mycelia was grown from conidial suspensions in
supplemented minimal media with 1 % glucose as the

20   carbon source, and 10 mM ammonium tartrate as the
nitrogen source for 16 hours, at 200 revs/min in an
orbital incubator at 37°C. The mycelia was
harvested by sieving through nylon fabric and
washing with chilled distilled water. The mycelia

25   was pressed dry between sheets of absorbent paper
and frozen in liquid nitrogen. The mycelia could be
stored at -70°C if necessary.

Transformation of A nidulans

30   The A nidulans transformation procedure was
developed from the methods of Ballance and Turner
(1983) and Cambell EJ et al. (1989, Curr. Genet,
16:53-56). Plasmids were transformed into A
nidulans strains by the co-transformation method. A

plasmid containing the argB gene or the N crassa
pyr4 gene was used to complement the argB2 or
pyrG89 mutation in A nidulans. Transformants were
obtained by selecting transformed protoplasts on
5   minimal media without the argB or pyr4 supplements.
By adding a five fold excess of another plasmid
with the complementing plasmid, some transformants
were obtained which also contained a genomic copy
of the other plasmid. Cellophane discs, 9 cm in
10   diameter were placed onto petri dishes of complete
medium. A conidial suspension was made up by
scraping 1 cm^2 of conidia from a fresh confluent A
nidulans plate and resuspending it into 3 ml of
distilled water. The conidial suspension was spread
15   onto 10 cellophane plates, 200 μl per plate, and
incubated at 37°C for 15 hours until the spores had
germinated. The cellophane's were then transferred
to two petri dishes each containing 15 ml of
novozyme 234 solution,(5 mg/ml novozyme 234 in 0.8
20   M $MgSO_4$, 10 mM PO_4 buffer pH 5.8) and incubated at
30°C for 1.5-2 hours. The cellophane's were washed
in 30 ml of solution A, (0.8 M $MgSO_4$ in 10 mM PO_4
buffer pH 5.8) and the wash solution and the
novozyme solution was sieved through a glass
25   scintillation funnel, size 1. The protoplasts were
centrifuged at 3000 revs/min for 10 minutes and
resuspended in 6 ml of solution B, (1.2 M sorbitol,
50 mM $CaCl_2$, 10 mM Tris pH 7.5).The protoplasts
were centrifuged at 2500 revs/min for 5 minutes and
30   resuspended in 6 ml of solution B and then
centrifuged at 2500 revs/min for 5 minutes. The
protoplast pellet was then resuspended in 400 μl of
solution B. 50 μl aliquots of the protoplast
suspension were transferred into plastic bijou's

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and up to 5 mg of plasmid DNA (1 mg/ml)was added.
The ratio of the plasmids transformed was 5:1,
(i.e., five times as much of the plasmid of
interest was used compared to the transforming
5   plasmid). 12.5 μl of solution C, (50% PEG 4000, 50
mM $CaCl_2$, 10 mM Tris pH7.5), was added to each
bijou of protoplasts and the suspension was gently
mixed. The cells were incubated on ice for 20
minutes and then 0.5 ml of solution C was added and
10   mixed with the cells. Immediately, 1 ml of solution
B was added, and the cells were mixed with 5 ml of
precooled (55°C) selection medium containing 1.2 M
sorbitol and 10 mM sodium nitrate as the sole
nitrogen source. An aliquot of untransformed
15   protoplasts was added to selection medium as a
control. The protoplasts were incubated at 30°C
overnight then at 37°C for 2-3 days until
conidiating colonies were obtained. Transformed
colonies were tested for their markers on selective
20   media before being experimented on.

Induction of A nidulans mycelia

     Conidial suspensions of A nidulans were grown
in supplemented minimal medium containing 0.1%
25   fructose as the carbon source, for 20 hours at 200
revs/min in an orbital incubator at 37°C. Inducing
chemical was added to the mycelia at 20 hours and
allowed to grow for a further 4 hours. The mycelia
was harvested as before. Uninduced mycelia was
30   allowed to grow for 24 hours in the medium
described above.

Protein extractions from A nidulans mycelia

     300 mgs of mycelia, frozen in liquid nitrogen,

was ground in a chilled pestle and mortar with an
equal amount of acid washed sand for 20 seconds. 1
ml of extraction buffer, (0.1 M Tris pH 7.5), was
added and the mycelia ground for a further 30

5   seconds. Another 1 ml of extraction buffer was
added and the mycelia was ground again for 30
seconds. 1 ml of the ground mycelia mixture was
transferred into eppendorfs and centrifuged at
13,000 revs/min for 5 minutes in a microcentrifuge.

10 The supernatant was transferred to new eppendorfs
and the protein concentration estimated using the
Bradford's assay.

DNA extractions from A nidulans mycelia

15   300 mgs of mycelia was ground in a precooled
pestle and mortar in liquid nitrogen until a fine
powder was obtained. The mycelia was transferred to
a 30 ml corex tube containing 7 ml of DNA
extraction buffer and then 7 ml of phenol was added

20 and mixed. 7 ml of chloroform: iaa was added and
the extraction mixture was gently mixed for 10
minutes. The mixture was centrifuged at 14,000
revs/min for 30 minutes and then the supernatant
was carefully transferred into several 1.5 ml

25 eppendorf tubes. The supernatant was extracted with
an equal volume of phenol, then phenol/ chloroform,
then chloroform. 100 μl of heat treated RNAse,(5
mg/ml), was added and incubated at 37°C for 30
minutes. Two volumes of ethanol and 0.1 volumes of

30 3 M sodium acetate was added and mixed and
incubated at room temperature for 10 minutes. The
DNA was pelleted by centrifugation at 13,000
revs/min for 10 minutes in a microcentrifuge and
then washed in 70% ethanol and dried at room

temperature for 30-60 minutes. The DNA was redissolved in TE or sterile distilled water at a concentration of 1 mg/ml.

5

RESULTS

(A)   **The regulator (alcR) constructs**
      Experiments have established that the alcR

10   regulator constructs function in A nidulans.   The constitutive promoters drive expression of the alcR gene to produce regulator protein.

      The alcR constructs were transformed into A

15   nidulans strain alcR125 (alcR⁻A⁺) using the above standard techniques.   Strain alcR125 is a null mutant strain which carries a point mutation in alcR and cannot express the regulator protein. Expression of the alcR gene introduced on the

20   regulator construct was tested by assaying alcohol dehydrogenase I (ADH1), since the alcR gene product is known to switch on the alcA gene coding for ADH1.   ADH1 activity was restored in the presence of inducer, demonstrating that the introduced alcR

25   regulator construct was functional in A nidulans. The alcR gene product may also be assayed directly by Western blotting of proteins using antibodies raised against purified alcR gene product expressed in E coli.   The transformed strain was back-crossed

30   to the parent strain to determine whether the regulator construct had integrated heterologously into the genome.   This experiment showed that the restoration of ADH1 activity was due to the presence of the regulator construct and not

25

reversion of the point mutation in alcR125. As a
control comparison, the alcR cDNA was transformed
into A nidulans without an associated promoter.

5   Further details of these experiments are given
below.

(i) The transformation of alcR125 A nidulans with
the alcR cDNA regulator construct:

10

        The alcR125, argB2 strain of A nidulans was
co-transformed with the p35SalcR/pUC and pILJ16
plasmids using the PEG method. The p35SalcR/pUC
plasmid contains the alcR cDNA between the 35S

15   promoter and the nos transcription terminator. The
pILJ16 plasmid contains a genomic copy of the argB2
gene from A nidulans and can complement argB2
mutations in A nidulans.

20   Five independent A nidulans transformants were
obtained which were able to grow to wild type
levels on media containing ethanol as the sole
source of carbon. The ability of a strain to grow
on ethanol-containing media is the phenotype of a

25   functional alcR gene. The transformants varied in
their ability to grow on ethanol , presumably the
result of the genomic position of the alcR
regulatory construct.

30   (ii) Back-crossing of the alcR+ transgenic A
nidulans strains with an alcR+ wild type strain:

        Two of the alcR+ transformants were back-
crossed with an alcR+ wild type strain and the

progeny tested for their ability to grow on
ethanol-containing media. The alcR+ wild type
strain was also wild type for spore colour. Two
hundred of the progeny obtained from the mature

5   cleistothesium were grown on ethanol-containing
media and scored for their alcR phenotype. The
ratio of alcR$^+$ to alcR$^-$ phenotype found in the
progeny was 3:1. The ratio of the green to yellow
phenotype in the same two-hundred progeny was found

10   to be 1:1.

(iii) Back-crossing of the alcR+ transgenic A
nidulans strains with an alcR125 strain:

15   The above two alcR+ transformants were
crossed with an alcR125 strain and the progeny
obtained were scored for alcR phenotype. The
alcR125 strain also had the panto mutation. Two
hundred progeny were grown on ethanol-containing

20   media and were found to exhibit an alcR$^+$ to alcR$^-$
ratio of 1:1. The ratio of the panto mutation to
wild type gene was also found to be 1:1.

25   **(B)   The chimeric promoter**
       The generation of stably transformed wild type
A nidulans with the alcA-CAT reporter construct
established that the chimeric promoter is operative
in A nidulans. Functioning of the alcA chimeric

30   promoter introduced on the reporter construct was
tested by assaying expression of the CAT gene.
Initiation of CAT gene expression under inducing
conditions demonstrated that the chimeric alcA:35S
promoter was functioning correctly.

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The alcA-CAT reporter construct was
transformed into a wild type A nidulans strain
(alcR⁺A⁺). The wild type strain has a normal alcR
gene producing the regulator protein necessary for
5   activation of the alcA promoter in the presence of
inducer.

Stably transformed A nidulans was obtained by
co-transforming a pyrG89 strain with the pDJB3 and
10   palcA-CAT/pUC plasmids using the PEG method.
.Plasmid palcA-CAT/pUC contains the CAT reporter
gene between the alcA:35S chimeric promoter and the
nos transcription terminator. Two independent
transformants (GA2-1 and GA2-5) were obtained which
15   contained uninterrupted copies of the alcA-CAT
construct. This was shown by probing genomic DNA,
restricted with HindIII, with the 0.7 kb SalI
fragment of the CAT gene.

20   The CAT activity of the alcA-CAT A nidulans
transformants was measured when they were grown
under a variety of conditions. Figure 4 is a graph
showing CAT expression from the GA2-1 and GA2-5
transformants (wild type with alcA-CAT construct)
25   growing in 0.1% fructose (F) or 1% glucose (G) in
the absence or presence of 50 mM ethyl methyl
ketone as inducer (I). The CAT activity of the
alcA-CAT reporter construct in the transformed A
nidulans strains was found to be high only when the
30   transformants were grown in 0.1% fructose and in
the presence of inducer. The induction of CAT was
prevented when the transformants were grown in 1%
glucose even in the presence of inducer indicating
the carbon catabolyte repression function of the

alcA promoter was retained. The two transformants,
GA2-1 and GA2-5, exhibited different CAT induction
abilities, most likely due to the differing genomic
positions of the alcA-CAT reporter constructs.

5

Transformant GA2-1 was found to be over three
times more inducible than transformant GA2-5 when
50 mM ethyl methyl ketone was used as the inducer.
Cyclohexanone was found to induce CAT expression in
10   the GA2-1 transformant. A concentration curve
showed that cyclohexanone had an optimal inducing
ability on the GA2-1 transformant when used at 25
mM. Figure 5 is a graph showing the effect of
cyclohexanone concentration (mM) on CAT expression
15   in the transformant GA2-1 growing in 0.1% fructose
(F) or 1% glucose (G).

Stably transformed A nidulans was also
obtained by co-transforming the pCaMVCN plasmid
20   (35S-CAT construct) with the pDJB3 plasmid into the
wild type strain using the PEG method. The plasmid
pDJB3 contains the pyr4 gene from N crassa, which
compliments the pyrG89 mutation in A nidulans. One
independent A nidulans transformant (GCMV-11) was
25   obtained which was found to contain the 35S-CAT
construct in uninterrupted form when genomic DNA,
restricted with HindIII, was probed with the 0.7 kb
SalI fragment of the CAT gene, in a Southern
hybridisation. Digestion of the genomic DNA with
30   XhoI and hybridisation with the 0.7 kbp SalI
fragment of the CAT gene revealed multiple copies
of the 35S-CAT sequence in the transformant. The
function of the 35S promoter was tested by
measuring the CAT expression obtained from the

35S-CAT <u>A</u> <u>nidulans</u> transformant. Figure 6 is a
graph showing CAT expression from the GA2-5
transformant (wild type with <u>alcA</u>-CAT construct)
and the GCMV-11 transformant (wild type with

5   35S-CAT construct) growing in 0.1% fructose (F) or
1% glucose (G) in the absence or presence of 50 mM
ethyl methyl ketone as inducer (I). The expression
of CAT in the 35S-CAT <u>A</u> <u>nidulans</u> transformant was
found to be at a much lower level than that of the

10   <u>alcA</u>-CAT <u>A</u> <u>nidulans</u> transformants.

.(C)   **The gene expression cassette**

The following experiments established that·the

15   gene expression cassette (comprising the <u>alcR</u>
regulator construct and the <u>alcA</u> reporter
construct) functions in <u>A</u> <u>nidulans</u>.

The <u>alcA</u> construct was transformed alone into

20   mutant <u>A</u> <u>nidulans</u> strain <u>alc500</u> (<u>alcR</u>⁻<u>A</u>⁻). This
mutant contains a deletion extending over both the
<u>alcR</u> and the <u>alcA</u> genes: hence the reporter protein
is not naturally present. There was a very low
rate of CAT gene expression. Thus, the <u>alcA</u>

25   construct was not functional in the absence of <u>alcR</u>
regulator protein. As a control comparison, the
pCaMVCN plasmid (containing the CAT gene under the
control of the constitutive CaMV 35S promoter) was
transformed into the <u>A</u> <u>nidulans</u> deletion strain

30   <u>alc500</u>.

The regulator <u>alcR</u> construct and the <u>alcA</u>
reporter construct were then co-transformed into
the <u>alcR</u>⁻<u>A</u>⁻ deletion strain <u>alc500</u>. Any expression

of the CAT reporter gene is induced by the <u>alcR</u>
regulator protein produced by the regulator
construct, without interference from a wild-type
regulator protein. Transformants were grown either
5   on fructose or glucose and subjected to inducing
conditions (4 hours in the presence of ethyl methyl
ketone, EMK) or non-inducing conditions (in the
absence of EMK). Results of the CAT assays are
shown in Table 1.

10

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TABLE 1

CAT assays

</div>

EMK (INDUCER)	CARBON SOURCE	μg CAT/mg protein
YES	fructose	5.67/4.58
NO	fructose	0.12/0.08
YES	glucose	0.01/0.00
NO	glucose	0.07/0.00

The results show that the CAT reporter gene is
correctly expressed under conditions which would
25   normally induce expression of the ADH1 gene: that
is, growth on fructose in the presence of inducer.

Growth on glucose results in poor expression
of the CAT reporter gene. Glucose is known to
30   repress ADH1 expression in wild type <u>A nidulans</u>
strains.

        The alcA:35S chimeric promoter is inducible.
The promoter drives expression of the CAT reporter
gene when the regulator protein and inducer are
present. Thus the gene expression cassette
5   (comprising the alcR and alcA constructs) is
functional and chemically-inducible.

        Similar studies were conducted using a
construct with the CaMV promoter alone fused to
10   CAT. In these experiments, low levels of CAT
activity were detected following growth on glucose
and were not induced by treatment with inducer.

        Expression vectors lacking the alcR regulator
15   gene were also transformed into A nidulans with the
alcA constructs. There was only a low level of CAT
gene expression, showing that it is the regulator
gene which controls the expression of the reporter
gene, through the action of the regulator protein
20   on the alcA fusion promoter.

                        EXAMPLE 4
                EXPRESSION IN PLANTS: TRANSIENT EXPRESSION IN
25   MAIZE PROTOPLASTS.

        The alcA-CAT and 35S-alcR constructs were
introduced into maize protoplasts to determine CAT
expression in transient assays after incubation in
30   the present of ethanol.

Isolation of protoplasts from maize
        Maize protoplasts were prepared from a
suspension culture of Black Mexican Sweet (BMS)

cells using the following method.

         The cells were harvested two days after a
    subculture and digested in E1 enzyme solution,
5   (2.0% cellulase RS, 0.2% pectolase Y23, 0.5M
    mannitol, 5mM CaCl2.2H2O, 0.5% MES pH5.6). 10 ml
    of enzyme solution was added to each gramme of
    tissue and incubated for 2 hours a 25°C in dim
    light while gently rotating. The digestion mixture
10 was sieved sequentially through 250 μm and 38 μm
    sieves, rinsing with wash buffer (0.358 M KCl, 1.0
    mM $NH_4.NO_3$, 5.0 mM $CaCl_2.2H_2O$, 0.5 mM KH_2PO_4
    pH4.8). The protoplasts were centrifuged at 700
    revs/min for 3.5 minutes and resuspended in 30 ml
15 of wash buffer twice. The protoplasts were counted
    using a Fuchs-Rosenthal haemocytometer. The
    protoplasts were then used for transient assays.

    Introduction of DNA into maize protoplasts
20   DNA was introduced into the protoplasts by
    the PEG uptake procedure. The protoplasts were
    resuspended at 2 X 10^6/ml in MaMg medium and
    aliquoted out at 0.5 ml per treatment. The
    protoplasts were heat shocked at 45°C for 5
25 minutes, then allowed to cool to room temperature
    ·for 10 minutes. A standard quantity of DNA, (at 1
    mg/ml), was added to the protoplast aliquots. 25
    μg of the control plasmid was added to an aliquot
    of protoplasts; the amount of other plasmids added
30 varied according to their size. 0.5 ml of PEG
    solution at 45°C was added and mixed gently with
    the protoplasts. The protoplasts were incubated at
    room temperature for 20 minutes then 5 ml of KCl
    solution was added, 1 ml at a time. The

protoplasts were incubated at room temperature for
10-15 minutes and then were centrifuged at 700
revs/min for 3.5 minutes. The supernatant was
removed and each sample was resuspended in 1.5 ml
5   of BMS + 9% mannitol and incubated at 25°C in the
dark for 22 hours.

The maize protoplasts were transformed with
the following constructs:
10   (a)   CaMV35S promoter fused to the chloramphenicol
           acetyl transferase gene (35S-CAT);
     (b)   alcA:35S chimeric promoter fused with the CAT
           gene (alcA-CAT);
     (c)   35S promoter fused to the alcR gene, plus
15   the alcA-CAT construct (alcA-CAT/35S-alcR);
     (d)   35S promoter fused to maize intron I and the
           alcR gene, plus the alcA-CAT construct
           (alcA-CAT/35S-I1-alcR).
           [Intron I from the maize adhI gene has
20   previously been shown (Callis et al, 1987,
           Genes and Development, 1:1183-1200) to
           increase expression from a number of genes in
           maize cells].
     Each plasmid combination was introduced into the
25   protoplasts in three separate aliquots.

Transient CAT expression assays
       Following incubation for 22 hours in media
containing 17 mM ethanol as an inducer of the alcR
30   gene product, protoplasts were harvested and
proteins extracted as follows.

       The protoplast samples were transferred to
eppendorfs and centrifuged at 13,000 revs/min for 3

minutes. 100 μl of 0.1 M Tris pH7.8 was added and
the cells were resuspended using a micro pipette.
The eppendorfs were vortexed for 30 seconds and
then centrifuged again at 13,000 revs/min for 5

5   minutes. 50 μl of the supernatant was used to
measure the CAT activity and the rest to estimate
the protein concentration using the Bradford's
method.

10   CAT activity was measured in samples of the
protein extract. CAT assays were undertaken
according to the Dupont method, where a
scintillation counter was used to measure transfer
of [^{14}C] acetyl chloramphenicol from the protein

15   extract into an immiscible scintillation fluid over
time.

      50 μl of protein extract was put in a plastic
disposable scintillation vial, together with 200 μl

20   of 1.25 mM chloramphenicol in 100 mM Tris pH7.8,
and 0.1 μCi of [^{14}C] acetyl CoA. 5 ml of
Econofluor, (950 ml/L toluene and 50 ml/L scint 7)
was gently overlaid onto the reaction mixture and
the vials were capped. The vials were counted

25   (cpm) sequentially in a scintillation counter for
30 seconds for a period of 1-2 hours. The cpm
values of the samples were plotted against time and
the gradient taken as a measure of the CAT activity
of the sample. The protein concentrations of the

30   samples were measured using Bradford's method. The
CAT activities of the samples were expressed as
14-[C]-acetyl chloramphenicol produced per minute
per mg of protein.

RESULTS

The data from this experiment are shown in
Figure 7. CAT gene expression is given as a
5   percentage of the CAT activity observed in cells
transformed with the 35S-CAT construct (production
of CAT under control of a constitutive promoter).
The CAT activity from the 35S-CAT construct was
expressed as 100% in order to compare results from
10   separate batches of protoplasts. The expression
from untransformed protoplasts is also shown.

Cells containing the alcA-CAT construct alone
showed a similar level of CAT activity as present
15   in control cells containing no DNA (the
untransformed protoplasts).

Cells containing the alcA-CAT and the
regulator constructs (35S-alcR and
20   35S-I1-alcR) show levels of CAT expression that are
7-fold and 16-fold higher respectively than cells
containing the alcA-CAT gene alone. This
establishes that expression of the CAT gene from
the alcA:35S promoter in maize protoplasts
25   incubated in media containing 17 mM ethanol (as the
inducer) is dependent on the presence of the alcR
gene. Also there is an apparent increase in CAT
expression in the presence of the 35S-I1-alcR
construct, presumably due to a higher level of
30   expression of alcR mediated by the inclusion of
maize intron I. The 35S-I1-alcR construct
increases CAT expression from the alcA-CAT
construct from 13% to 31% of that from the 35S-CAT
construct.

Further experiments with maize protoplasts
have shown that the plant gene expression cassette
is functioning correctly. For example, Figure 8 is
a graph showing the effect of the 35S-alcR
5   regulator construct on CAT expression from the
alcA-CAT construct in the presence of 17 mM
ethanol. A 35S-I1-CAT construct was used as the
positive control in this experiment.

10

EXAMPLE 5
TIME COURSE OF EXPRESSION IN A STABLY
TRANSFORMED TOBACCO PLANT

15
Construction of tobacco transformation vectors

Bin19 derivatives of the alcA-CAT construct
and 35S-alcR/alcA-CAT construct were produced using
standard procedures. Bin19 is a known plant
20   transformation vector (Bevan, 1984, Nucleic Acids
Research 12, 8711-8721).

The plasmid palcA-CAT/BIN contains the CAT
reporter gene between the alcA:35S chimeric
25   promoter and the nos transcription terminator. It
was constructed as follows: The 1.4 kbp HindIII
fragment from from the alcA-CAT/pUC plasmid
(containing the alcA:35S fusion promoter, the CAT
gene and the nos transcription terminator) was
30   cloned into the HindII site of the pJRli/BIN19
vector. Transformed colonies were transferred to
nylon membrane and were probed with the alcA-CAT
sequence. Positive colonies were shown to be
correct when miniprepped plasmid DNA digested with

HindIII gave a 1.4 kbp fragment.

The plasmid p35SalcR-alcA-CAT/BIN contains the
alcR cDNA between the 35S promoter and the nos
transcription terminator, and the CAT reporter gene
between the alcA:35S chimeric promoter and the nos
transcription terminator. It was constructed as
follows: Plasmid 35S-alcR/pUC was fully digested
with HindIII and partially digested with EcoRI.
The 3.5 kbp fragment was isolated and cloned into
the pJR1i/BIN19 vector which had been restricted
with EcoRI and HindIII. The 1.4 kbp HindIII
fragment containing the alcA-CAT sequence from
palcA-CAT/pUC was then cloned into the HindIII site
in the 35S-alcR/JR1i/BIN plasmid. The correct
plasmid was shown to contain the 1.4 kbp HindIII
fragment form the alcA-CAT sequence, and the 3.0
kbp and 0.5 kbp EcoRI fragments from the 35S-alcR
sequence. A 3.5 kbp partial EcoRI/HindIII fragment
could be seen in the same digest. The orientation
of the alcA-CAT sequence to the 35S-alcR sequence
in the final plasmid is estimated to be head to
tail.

Transformation of Agrobacterium tumefaciens
     The disarmed Agrobacterium tumefaciens strain
LBA4404 (pAL4404)(Hoekema et al, 1983, Nature 303,
179-180) was transformed with the above vectors
using the freeze-thaw method. A single colony was
grown up in 40 ml of LB medium at 28°C in an
orbital incubator at 200 revs/min overnight until
the culture reaches an $O.D._{580}$ of 0.5-1.0. The
cells were resuspended in 1 ml of ice cold 20 mM
$CaCl_2$ solution, and then dispensed into prechilled

eppendorfs, 100 μl per tube. DNA was added to the
cells, 0.1 μg/100 μl of cells, and then the cells
were frozen in liquid nitrogen. The cells were
then thawed at 37°C in a water bath for 5 minutes.

5   1 ml of LB medium was added to each tube and the
cells were incubated at 28°C for 2-4 hours with
gentle shaking to allow the bacteria to express the
antibiotic resistance genes. The cells were
centrifuged for 30 seconds at 13,000 revs/min in a

10   microcentrifuge and the supernatant was discarded.
The cells were resuspended in 100 μl of LB medium.
The cells were spread onto LA agar plates
containing 50 μg/ml kanamycin, or other antibiotic
selection afforded by the introduced plasmid. The

15   plates were incubated at 28°C for 2 days, when
colonies were likely to appear.

Plasmid minipreps from Agrobacterium tumefaciens
       Single colonies were grown in 20 ml of LB

20   medium containing antibiotic as the selective agent
overnight at 28°C in an orbital incubator. The
cells were centrifuged at 3000 revs/min for 5
minutes and the pellet was resuspended in 0.5 ml of
miniprep solution, (5 mg/ml lysozyme in 50 mM

25   glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). The
cells were incubated on ice for an hour, and then 1
ml of alkaline SDS (0.2 M NaOH, 1% SDS) was added.
After incubation on ice for 10 minutes, 0.75 ml of
3 M sodium acetate was added, and the mixture was

30   left on ice for 30 minutes. The lysis mixture was
centrifuged at 15,000 revs/min for 10 minutes at
4°C, and the supernatant was transferred to 15 ml
corex tubes. 5 ml of cold ethanol was added and
the tubes were stored at -70°C for 30 minutes. The

tubes were then centrifuged at 15,000 revs/min for
15 minutes at 4°C and the supernatant was removed.
The pellet was dissolved in 0.5 ml of T.E. and
transferred to eppendorfs. The DNA solution was
5   extracted with an equal volume of phenol/chloroform
three times and once with an equal volume of
chloroform. The DNA was precipitated by adding 1
ml of ethanol and incubating at -70°C for 30
minutes. After centrifugation at 13,000 revs/min
10   for 15 minutes in a microcentrifuge the DNA pellet
was washed with 70% ethanol, dried at room
temperature for a few minutes, and redissolved in
50μl T.E. 20μl of DNA solution was used per
restriction digest. Generally, a 2-3 fold increase
15   in restriction enzyme and at least 4 hours
digestion was required for the Agrobacterium DNA to
be digested adequately.

Tobacco transformations
20   Transgenic tobacco was generated by the leaf
disc method using transformed Agrobacterium
tumefaciens. About 20 tissue culture grown plats
were required per transformation. The plants were
about 3-4 weeks old and were grown on M.S medium
25   without antibiotics. All manipulations were
carried out in sterile hoods using sterile
implements.

Leaves were cut from the tissue culture
30   plants, placed on NBM medium in petri dishes, and
incubated overnight in a plant growth room. The
transformed Agrobacterium strain was grown up
overnight in 100ml of LB containing kanamycin at 50
μg/ml. The next day the culture was centrifuged at

3000 revs/min for 10 minutes and resuspended in an
equal volume of MS solution. 20 ml of
Agrobacterium solution was placed in 9 cm petri
dishes. Leaf discs were made form the leaves,
5   using a sterile scalpel, and were put into the
Agrobacterium solution in the petri dishes for 20
minutes. The leaf pieces were then transferred to
the NBM plates and incubated overnight in a plant
growth room. After 48 hours the leaf discs were
10   transferred to NBM medium containing carbenicillin
at 500 μg/ml and kanamycin at 100 μg/ml in neoplant
pots. The pots were incubated in a plant growth
room for 4-6 weeks. Shoots emerging from callous
tissue were transferred to MS medium containing
15   carbenicillin at 200 μg/ml and kanamycin at 100
μg/ml in neoplant pots, (7 to a pot). After 3
weeks, shoots that had rooted were transferred to
fresh MS medium and grown on until they were about
5 cm in height. Extra cuttings were taken at this
20   stage. The plants were then transferred to compost
in 13 cm pots and sealed in polythene bags to
prevent dehydration for the first few weeks.

Induction of transgenic tobacco plants
25   Cyclohexanone was applied to the plant leaves
as a paint. The cyclohexanone paint consisted of a
10% solution of 950 g/L cyclohexanone, 35g/L
syperonic NDE 1800, 16.5 g/L Tween 85 in thickener.
Both surfaces of the leaf were applied with paint
30   at 100 μl solution per 16-25 cm^2 of leaf surface,
on days 0 and 2. Leaf tissue was harvested prior
to inducer application and at appropriate times
after the second application of paint. 1 cm^2 of
leaf tissue was removed with a sterile scalpel, put

in an eppendorf, and immersed in liquid nitrogen.
CAT assays were undertaken within 4 hours of leaf
sampling.

5   Protein extractions from tobacco leaf tissue
        The leaf tissue was allowed to defrost at room
temperature for several minutes after storage in
liquid nitrogen. A sterile micropestle was used to
grind the leaf tissue in the eppendorf for 30
10   seconds and then 100 μl of 100 mM Tris pH 7.8 was
added. The plant mixture was then vortexed for 20
seconds and centrifuged at 13,000 revs/min for 5
minutes. The supernatant was transferred to a new
eppendorf. 50 μl of leaf extract was used for a
15   CAT assay and the rest was used to determine the
protein concentration wit the Bradford's method.

        CAT assays
        CAT assays were undertaken according to the
20   Dupont method as described in Example 4.

        RESULTS

25   (A) Generation of stably transformed tobacco with
             the alcA-CAT reporter construct
        The plasmid palcA-CAT/BIN was used to produce
transgenic tobacco plants. Forty-four independent
tobacco transformants were obtained, of which
30   twenty-three were shown to contain the alcA-CAT
construct when genomic DNA was amplified using PCR
with the oligo's C3 and A3. Four plants were shown
to contain the construct in uninterrupted form when

genomic DNA, restricted with <u>Hind</u>III, was probed
with the 0.7 kb <u>Sal</u>I of the CAT gene.

(B)　Generation of stably transformed tobacco with
the alcA-CAT reporter and the alcR-cDNA
regulator cassette

The plasmid p35S<u>alc</u>R-<u>alc</u>A-CAT/BIN was used to
produce transgenic tobacco plants. Twelve
independent tobacco transformants were obtained,
and analysed for the presence of the <u>alc</u>R
regulatory and <u>alc</u>A-CAT reporter constructs using
PCR. Ten of these plants were shown to contain the
<u>alc</u>R regulatory construct using the oligos, L4 and
L6, in PCR. One of those plants, D3, was shown to
contain the <u>alc</u>A-CAT reporter construct by PCR
using the oligos, A3, and C3.

(C)　Analysis of the CAT expression found in the
transgenic tobacco before induction

Leaf tissue was used to measure the CAT
activity of the transgenic plants. All of the <u>alc</u>A-
CAT transgenic plants were found to exhibit low CAT
activity levels. The CAT expression levels varied
between individual plants but none were found to
have higher CAT expression levels than those of
the control tobacco plants. None of the <u>alc</u>R
regulatory/<u>alc</u>A-CAT reporter transgenic tobacco
plants were found to have high CAT expression
levels. The CAT activities were low and were not
above those of the control tobacco.

(D)   The effect of cyclohexanone on the CAT
       activities of the plants

None of the alcA-CAT transgenic plants were
found to have high CAT expression levels after the
5   application of the cyclohexanone paint to the
leaves. The CAT expression levels varied between
plants, but none were found to have expression
levels higher than those of the control tobacco
plants. Of the 35S-alcR/alcA-CAT transgenic
10   tobacco plants, one, D3, was found to exhibit a
high CAT expression level after application of
cyclohexanone to its leaves. The level was much
higher than that found in control tobacco plants.

15   (E)   Time course experiment following CAT activity
       after application of cyclohexanone

The plant known as D3 was shown by PCR to
contain both the alcR regulatory and the alcA-CAT
reporter gene fusions (ie the entire gene
20   expression cassette).

This experiment was a time course analysis of
plant D3 compared with a plant containing only the
alcA-CAT construct (known as S34) and with an
25   untransformed control (known as T1), following
treatment with a formulated leaf paint containing
the alcR inducer, cyclohexanone. The latter has
been shown previously to be a potent inducer of
ADHI expressed from the alcA gene in Aspergillus
30   cells.

The data obtained from this analysis are shown
in Figure 9: at each time point, results are given
in the order S34:D3:T1. Leaves were treated with

cyclohexanone leaf paint on day 0 and day 2.
Samples of leaf tissue were taken immediately prior
to each treatment (0-0 and 2-0 in Figure 9) and
thereafter at seven intervals up until day 3.

5   Protein extracts were made from the leaf samples
and CAT activity determined in the samples using
the diffusion assay described above.

       Following painting with cyclohexanone, CAT
10   activity increases in the leaves of plant D3
(containing the 35S-alcR/alcA-CAT genes), reaching
a peak at 160 minutes after the second inducti⁖
treatment, and thereafter declines (Figure 9).

15   In contrast, treatment with cyclohexanone had
no effect on the untransformed plant (T1) or the
plant containing only the alcA-CAT gene (S34). In
these plants, CAT activity remained at about 5-20%
of the maximum activity seen in the
20   35S-alcR/alcA-CAT leaves.

       This experiment established that in stably
transformed plants containing the gene expression
cassette 35S-alcR/alcA-CAT, high levels of
25   expression of the reporter gene (CAT) are only
obtained in the presence of the alcR regulator gene
product and following exogenous treatment with the
inducing chemical, cyclohexanone. The inducer
cyclohexanone has no effect on CAT gene expression
30   plants containing alcA-CAT alone.

(F)   The effect of wounding on the CAT expression
      in the plants

      Leaves of the plants were lacerated with a
razor blade and the effect on CAT expression was

5   measured. Samples of leaf tissue taken 240 minutes
after laceration showed no increase in CAT activity
in any of the plants (ie no induction of gene
expression). Therefore wounding was found to have
no effect on the alc expression cassette by this

10   method.

(G)   The effect of iso-propanol on the CAT
      expression of the plants

      Iso-propanol is a weak inducer and a substrate

15   for alcA, whereas cyclohexanone is an inducer but
not a substrate (Creaser et al, 1984, Biochem J,
255:449-454). Iso-propanol has no apparent effect
on the alc expression cassette.

20   The leaves of the plants were painted with a
10% iso-propanol solution at 0 and 60 minutes and
the CAT expression of leaf samples was measured at
240 minutes. The CAT expression found in the alcA-
CAT and alcA-CAT/35S-alcR transgenic plants at 0

25   minutes was low as it was in the control
untransformed tobacco plants. After 240 minutes no
significant increase in CAT activity was detected
in any of the plant types.

CLAIMS

1. A chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which encodes a regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

2. A plant gene expression cassette as claimed in claim 1 wherein the regulator sequence is derived from the alcR gene.

3. A plant gene expression cassette as claimed in claim 1 or claim 2 wherein the inducible promoter is derived from the alcA gene promoter.

4. A plant gene expression cassette as claimed in claim 3 wherein the inducible promoter is a chimeric promoter.

5. A plant cell containing a gene expression cassette as claimed in claim 1.

6. A plant cell as claimed in claim 5 wherein the gene expression cassette is stably incorporated in the plant's genome.

7. A plant tissue comprising a plant cell as claimed in claim 6.

8.   A plant comprising a plant cell as claimed in
      claim 6.

9.   A plant derived from a plant as claimed in
      claim 8.

10.   A seed derived from a plant as claimed in
      claim 8 or claim 9.

11.   A method for controlling plant gene expression
      comprising transforming a plant cell with a
      chemically-inducible plant gene expression
      cassette which has a first promoter
      operatively linked to a regulator sequence
      which encodes a regulator protein, and an
      inducible promoter operatively linked to a
      target gene, the inducible promoter being
      activated by the regulator protein in the
      presence of an effective exogenous inducer
      whereby application of the inducer causes
      expression of the target gene.

12.   A chimeric promoter comprising an upstream
      region containing a promoter regulatory
      sequence and a downstream region containing a
      transcription initiation sequence,
      characterised in that said upstream and
      downstream regions are heterologous.

13.   A chimeric promoter as claimed in claim 12
      wherein the regulatory sequence is derived
      from the alcA gene promoter.

14.   A chimeric promoter as claimed in claim 12 or
      claim 13 wherein the transcription initiation
      sequence is derived from the core promoter

region of a promoter which is active in plant
cells.

15.   A chimeric promoter as claimed in claim 12 or
        claim 13 which is chemically inducible.

16.   A plant cell containing a chimeric promoter as
        claimed in claim 12.

17.   A plant cell as claimed in claim 16 wherein
        the chimeric promoter is stably incorporated
        in the plant's genome.

18.   A plant tissue comprising a plant cell as
        claimed in claim 17.

19.   A plant comprising a plant cell as claimed in
        claim 17.

20.   A plant derived from a plant as claimed in
        claim 19.

21.   A seed derived from a plant as claimed in
        claim 19 or claim 20.

22.   A plant cell as claimed in claim 5 or claim
        16 which is a monocotyledonous plant cell.

23.   A plant cell as claimed in claims 22 which is
        a maize cell.

24.   A plant cell as claimed in claim 5 or claim 16
        which is a dicotyledonous cell.

25.   A plant cell as claimed in claim 24 which is a
        tobacco cell.

FIG. 1 (1/2)

Production of 35S Regulator Construct

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FIG.1 (2/2)
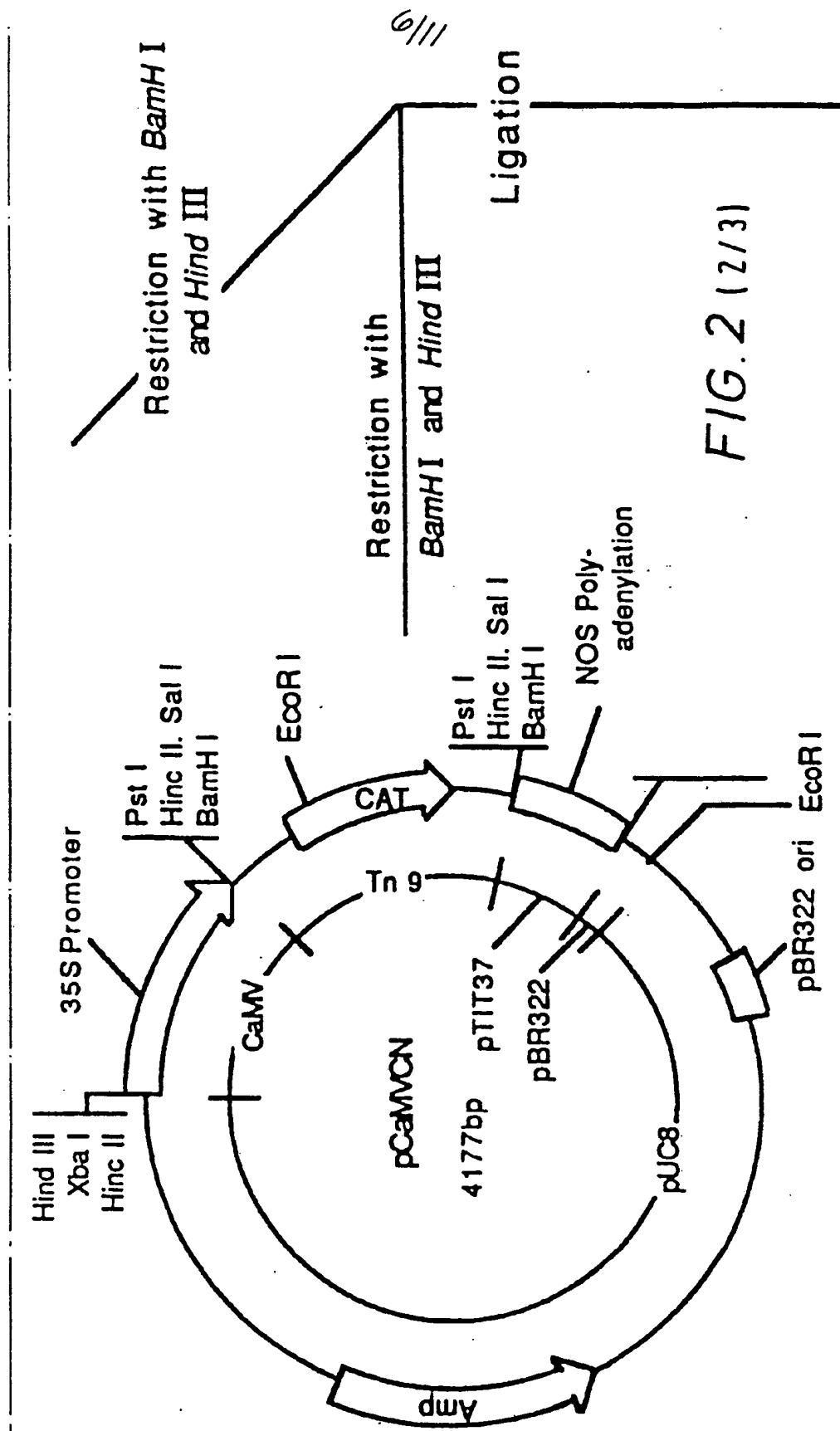
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FIG. 2 (1/2)

Production of pNOS Regulator Construct

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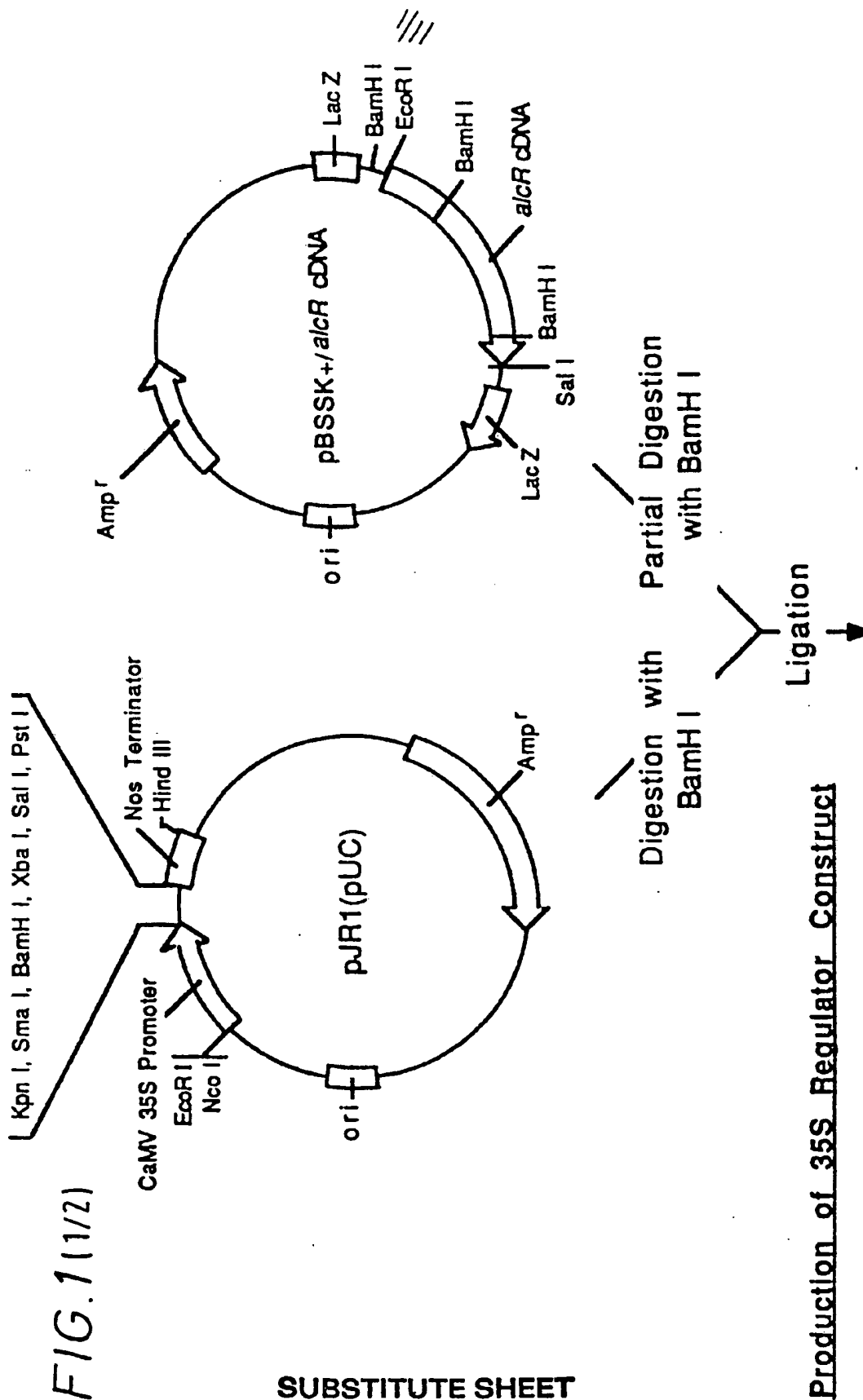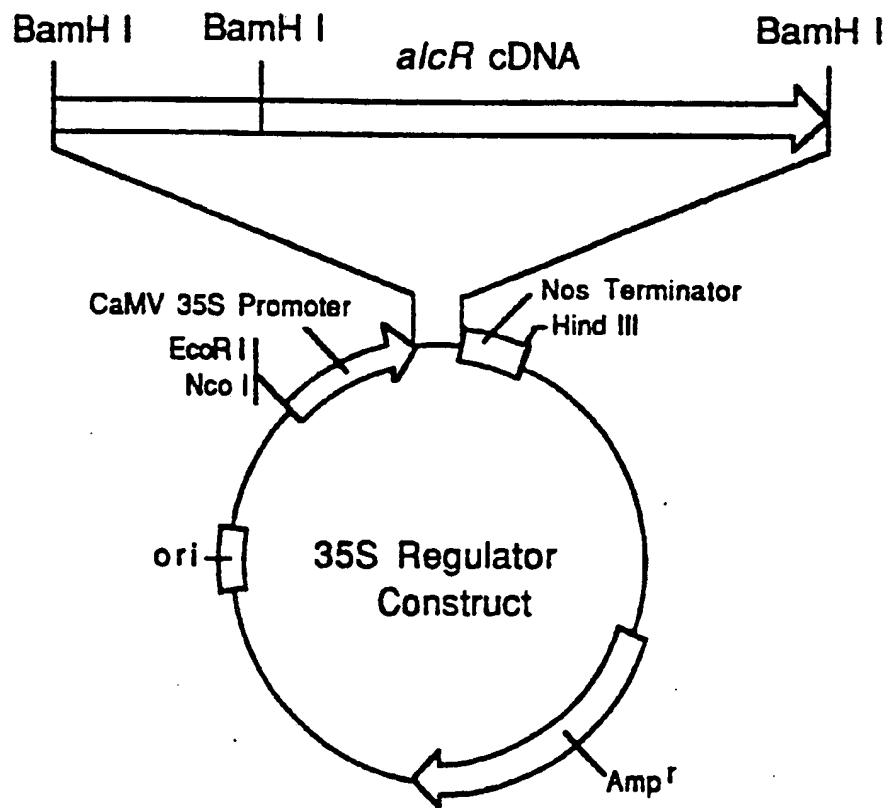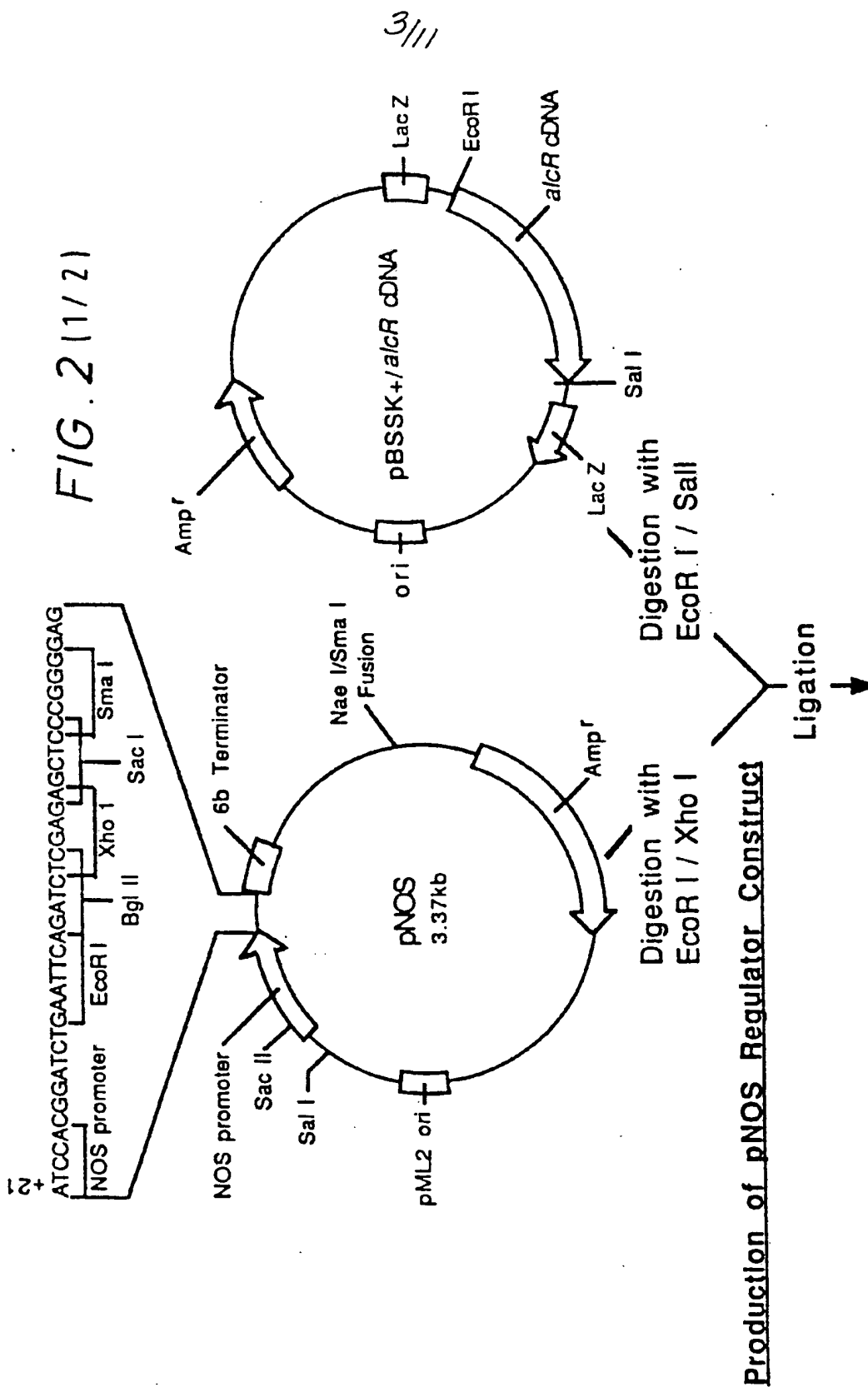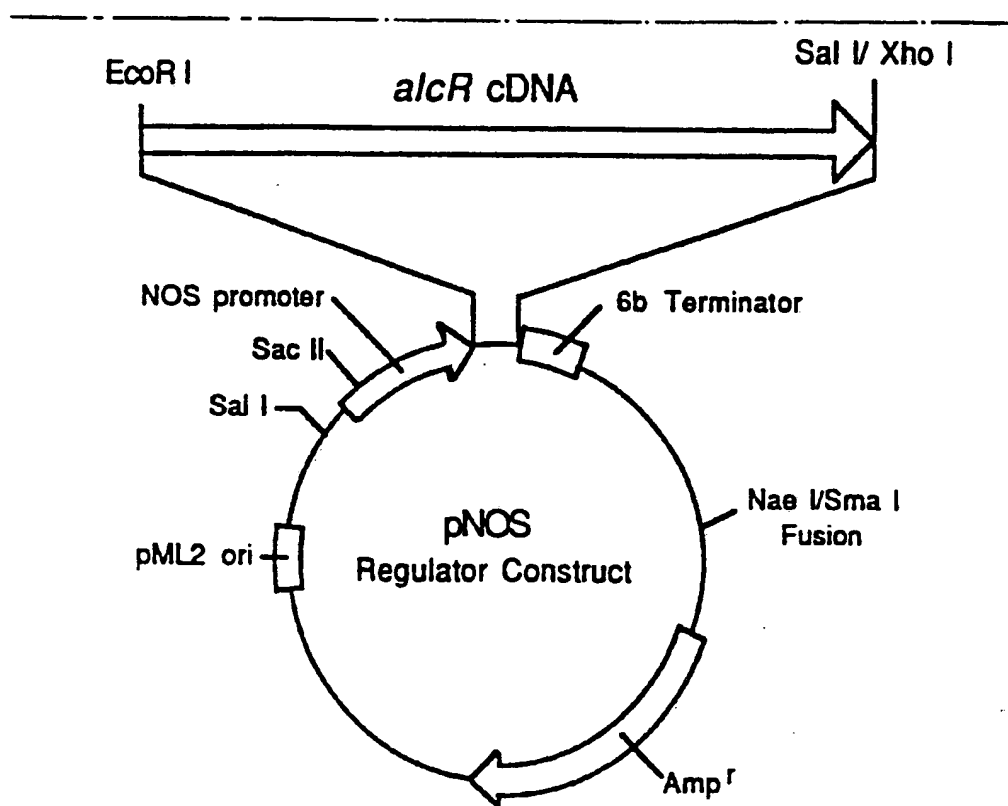
FIG.2 (2/2)

FIG. 3 (1/3)
Production of Reporter Construct

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FIG. 2 (2/3)

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FIG. 3 (3/3)

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FIG. 5

FIG. 4

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FIG. 8

FIG. 6

FIG.7

CAT EXPRESSION LEVEL (AS% OF 35S-CAT LEVEL)

110
100
90
80
70
60
50
40
30
20
10
0

UNTRANSFORMED PROTOPLASTS

35S-CAT

alcA-CAT

alcA-CAT/35S - alcR

alcA-CAT/35S-I-alcR

FIG.9